IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: TAKAKURA=6A

In re Application of:	)	Art Unit:
	)	
Hikaru TAKAKURA et al.	)	Examiner:
	)	
Serial No.: NOT YET ASSIGNED	)	Washington, D.C.
	)	
Filed: ON EVEN DATE HEREWITH	)	March 6, 2002
	)	
For: THERMOSTABLE PROTEASE	)	

PRELIMINARY AMENDMENT

Honorable Commissioner of Patents
Washington, D.C. 20231

Sir:

Contemporaneous with the filing of this case and prior to calculation of the filing fee, kindly amend as follows:

IN THE SPECIFICATION

After the title please insert the following paragraph:

-- CROSS-REFERENCE TO RELATED APPLICATION

The present application is a divisional of application 09/445,472, filed December 8, 1999, which is the national stage under 35 U.S.C. 371 of PCT/J98/02465, filed June 4, 1998, which claims priority from JP 151969/1997, filed June 10, 1997.--

Please replace the paragraph beginning at page 5, line 11 with the following rewritten paragraph:

--The third invention of the present invention is a

gene to be used for producing a thermostable protease derived from a hyperthermophile by genetic engineering technique, characterized in that the gene encodes an amino acid sequence represented by formula I:

SIG-Ala-Gly-Gly-Asn-PRO [I] (SEQ ID NO:30)

wherein SIG represents an amino acid sequence of a signal peptide derived from a subtilisin, PRO represents an amino acid sequence of a protein to be expressed. Preferably, SIG is the amino acid sequence represented by the SEQ ID NO:3 of the Sequence Listing. Preferably, PRO is an amino acid sequence of a hyperthermostable protease derived from a hyperthermophile, more preferably, an amino acid sequence of a protease derived from *Pyrococcus furiosus*.--

Please replace the paragraph beginning at page 9, line 3, with the following rewritten paragraph:

--Figures 2-5 compare the amino acid sequences of Protease PFUL (SEQ ID NO:6), Protease TCES (SEQ ID NO:12) and a subtilisin (SEQ ID NO:31).--

Please delete the paragraphs at page 9, lines 5-10.

Please replace the paragraph beginning at page 16, line 8, with the following rewritten paragraph:

--Hydrolyzes succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-4-methylcoumarin-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) SEQ

ID NO:32 to generate a fluorescent substance (7-amino-4-methylcoumarin).--

Please replace the paragraph beginning at page 16, line 12, with the following rewritten paragraph:

--Hydrolyzes succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (Suc-Ala-Ala-Pro-Phe-p-NA) SEQ ID NO:33 to generate a yellow substance (p-nitroaniline).--

Please replace the paragraph beginning at page 21, line 17, with the following rewritten paragraph:

--Hydrolyzes succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-4-methylcoumarin-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) SEQ ID NO:32 to generate a fluorescent substance (7-amino-4-methylcoumarin).--

Please replace the paragraph beginning at page 21, line 21, with the following rewritten paragraph:

--Hydrolyzes succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (Suc-Ala-Ala-Pro-Phe-p-NA) SEQ ID NO:33 to generate a yellow substance (p-nitroaniline).--

Please replace the paragraph beginning at the top of page 28, with the following rewritten paragraph:

--The protease expressed from the transformant exhibits enzymological properties equivalent to those of the protease expressed by *Bacillus subtilis* DB104/pSNP1 as described

above. The protease expressed by the transformant was purified. The analysis of the N-terminal amino acid sequence of the purified protease provided the amino acid sequence as shown in the SEQ ID NO:22 of the Sequence Listing. This sequence is identical with the sequence from position 133 to position 144 of the amino acid sequence of Protease PFUS as shown in the SEQ ID NO:16 of the Sequence Listing, indicating that the mature Protease PFUS is an enzyme consisting of a polypeptide starting from this portion. The amino acid sequence of the mature Protease PFUS assumed from these results is shown in the SEQ ID NO:4 of the Sequence Listing.--

Please replace the paragraph beginning at page 31, line 8, with the following rewritten paragraph:

--Thus, it proved that the expression level of the protein of interest can be increased in a bacterium of genus *Bacillus* as a host by placing a peptide consisting of four amino acid residues Ala-Gly-Gly-Asn (SEQ ID NO:30) downstream the signal peptide of a subtilisin, fusing it to the N-terminus of the protein of interest and expressing the fused protein. In addition to subtilisin E (from *Bacillus subtilis*) which is used in the present invention, subtilisin BPN' from *Bacillus amyloliquefaciens* (Nucl. Acids Res., 11:7911-7925 (1983)), subtilisin Carlsberg from *Bacillus licheniformis* (Nucl. Acids Res., 13:8913-8926 (1985)) and the like are known as subtilisins produced by bacteria of genus *Bacillus*. The signal peptides from them can be preferably used for the present invention although

their amino acid sequences slightly vary each other. Various promoters which function in a bacterium of genus *Bacillus* can be used in place of the promoter from the subtilisin E gene which is used in the present invention for controlling expression.--

Please replace the paragraph beginning at page 33, line, with following rewritten paragraph:

--The molecular weight of the mature Protease PFUS obtained from *Bacillus subtilis* DB104/pNAPS1 can be precisely measured, for example, by using a mass spectrometer. It is found from the measured molecular weight and the N-terminal amino acid sequence of the mature Protease PFUS determined as described above that the protease is a polypeptide corresponding to Ala at position 133 to Thr at position 552 of the amino acid sequence as shown in the SEQ ID NO:16 of the Sequence Listing. Furthermore, a plasmid which expresses Protease PFUS lacking a polypeptide nonessential for its enzymatic activity can be constructed by introducing a termination codon in the vicinity of the portion encoding Thr at position 552 in the Protease PFUS gene contained in the plasmid pNAPS1. Specifically, a DNA fragment having a base sequence into which the intended termination codon is introduced can be obtained by PCR using the primer NPR544 which can introduce a termination codon (TGA) on the C-terminal side of the 544th amino acid residue encoding codon from the initiation codon in the Protease PFUS gene in the plasmid pNAPS1 (Ser) (the base sequence of the primer NPR544 is shown in the SEQ ID NO:28 of the Sequence Listing) and the primer NPFE81 which has the base sequence of the region upstream

from the NspV site in the gene (the base sequence of the primer NPFE81 is shown in the SEQ ID NO:29 of the Sequence Listing). A mutant plasmid containing the protease gene into which the mutation of interest is introduced can be then obtained by replacing the fragment for the corresponding region in the plasmid pNAPS1. This plasmid is designated as the plasmid pNAPSΔC. *Bacillus subtilis* DB104 transformed with this plasmid is designated as *Bacillus subtilis* DB104/pNAPSΔC.--

Please replace the paragraph beginning at page 36, line 9, with the following rewritten paragraph:

--Hydrolyzes succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-4-methylcoumarin-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) SEQ ID NO:32 to generate a fluorescent substance (7-amino-4-methylcoumarin).--

Please replace the paragraph beginning at page 36, line 13, with the following rewritten paragraph:

--Hydrolyzes succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (Suc-Ala-Ala-Pro-Phe-p-NA) SEQ ID NO:33 to generate a yellow substance (p-nitroaniline).--

Please replace the paragraph beginning at page 49, line 7, with the following rewritten paragraph:

--The activity of Protease PFUS was determined by spectroscopically measuring the amount of p-nitroaniline generated in an enzymatic hydrolysis reaction using Suc-Ala-Ala-

Pro-Phe-p-NA (Sigma) (SEQ ID NO:33) as a substrate. Briefly, an enzyme preparation to be measured for its enzymatic activity was appropriately diluted. 50 µL of 1 mM Suc-Ala-Ala-Pro-Phe-p-NA (SEQ ID NO:33) solution in 100 mM phosphate buffer, pH 7.0 was added to 50 µL of the diluted sample solution. Then, the reaction was allowed to proceed at 95°C for 30 minutes. After terminating the reaction by cooling on ice, absorbance at 405 nm was measured to calculate the amount of p-nitroaniline generated. One unit of the enzyme was defined as the amount of the enzyme which generated 1 µmole of p-nitroaniline per 1 minute at 95°C. The amount of enzyme protein expressed in the culture supernatant or the cells was calculated based on the measured enzymatic activity assuming the specific activity as 9.5 unit/mg protein of Protease PFUS.--

Please replace the paragraph at the bottom of page 53, with the following rewritten paragraph:

--N-terminal amino acid sequences of the purified enzyme preparations NAPS-1 and SPO-124ΔC were analyzed by automated Edman method using G1000A protein sequencer (Hewlett-Packard). Both of the N-terminal amino acid sequences of the two purified enzyme preparations were as shown in the SEQ ID NO:22 of the Sequence Listing. This sequence coincides with the sequence from position 133 to position 144 of the amino acid sequence of Protease PFUS as shown in the SEQ ID NO:16 of the Sequence Listing, indicating that both of NAPS-1 and SPO-124ΔC

are enzymes consisting of a polypeptide starting from this portion.--

Please replace the paragraph on page 53, line 13, with the following rewritten paragraph:

--Mass spectrometric analysis on the purified enzyme preparations NAPS-1 and SPO-124ΔC was carried out using API300 quadrupole triple mass spectrometer (Perkin-Elmer Sciex). Based on the estimated molecular weight of NAPS-1, 43,744 Da, it was demonstrated that the mature Protease PFUS produced by *Bacillus subtilis* DB104/pNAPS1 is an enzyme consisting of a polypeptide from Ala at position 133 to Thr at position 552 of the amino acid sequence of Protease PFUS as shown in the SEQ ID NO:16 of the Sequence Listing. Furthermore, based on the estimated molecular weight of SPO-124ΔC, 42,906 Da, it was demonstrated that the mature Protease PFUS produced by *Bacillus subtilis* DB104/pSPO124ΔC is an enzyme consisting of a polypeptide from Ala at position 133 to Ser at position 544 of the amino acid sequence of Protease PFUS as shown in the SEQ ID NO:16 of the Sequence Listing, i.e., the amino acid sequence as shown in the SEQ ID NO:1 of the Sequence Listing.--

REMARKS

The specification is amended to provide a "Cross-Reference to Related Applications" section and to provide consistency with the amended specification of the parent application.

Favorable consideration is respectfully requested.

Respectfully submitted,

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

The paragraph beginning at page 5, line 11 has been amended as follows:

The third invention of the present invention is a gene to be used for producing a thermostable protease derived from a hyperthermophile by genetic engineering technique, characterized in that the gene encodes an amino acid sequence represented by formula I:

SIG-Ala-Gly-Gly-Asn-PRO [I] (SEQ ID NO:30)

wherein SIG represents an amino acid sequence of a signal peptide derived from a subtilisin, PRO represents an amino acid sequence of a protein to be expressed. Preferably, SIG is the amino acid sequence represented by the SEQ ID NO:3 of the Sequence Listing. Preferably, PRO is an amino acid sequence of a hyperthermostable protease derived from a hyperthermophile, more preferably, an amino acid sequence of a protease derived from *Pyrococcus furiosus*.

The paragraph beginning at page 9, line 3, has been amended as follows:

Please replace the paragraph beginning at page 9, line 3, with the following rewritten paragraph:

Figures 2-5 compares the amino acid sequences of Protease ~~PFUS~~ PFUL (SEQ ID NO:6), Protease TCES (SEQ ID NO:12) and a subtilisin (SEQ ID NO:31).

Delete on page 9, lines 5-10 as follows:

~~Figure 3 compares the amino acid sequences of Protease PFUS, Protease TCES and a subtilisin.~~

~~Figure 4 compares the amino acid sequences of Protease PFUS, Protease TCES and a subtilisin.~~

~~Figure 5 compares the amino acid sequences of Protease PFUS, Protease TCES and a subtilisin.~~

The paragraph beginning at page 16, line 8, has been amended as follows:

Hydrolyzes succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-4-methylcoumarin-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) SEQ ID NO:32 to generate a fluorescent substance (7-amino-4-methylcoumarin).

The paragraph beginning at page 16, line 12, has been amended as follows:

Hydrolyzes succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (Suc-Ala-Ala-Pro-Phe-p-NA) SEQ ID NO:33 to generate a yellow substance (p-nitroaniline).

The paragraph beginning at page 21, line 17, has been amended as follows:

Hydrolyzes succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-4-methylcoumarin-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) SEQ ID NO: 32 to generate a fluorescent substance (7-amino-4-methylcoumarin).

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The paragraph beginning at page 31, line 8, has been amended as follows:

Thus, it proved that the expression level of the protein of interest can be increased in a bacterium of genus

Bacillus as a host by placing a peptide consisting of four amino acid residues Ala-Gly-Gly-Asn (SEQ ID NO:30) downstream the signal peptide of a subtilisin, fusing it to the N-terminus of the protein of interest and expressing the fused protein. In addition to subtilisin E (from *Bacillus subtilis*) which is used in the present invention, subtilisin BPN' from *Bacillus amyloliquefaciens* (Nucl. Acids Res., 11:7911-7925 (1983)), subtilisin Carlsberg from *Bacillus licheniformis* (Nucl. Acids Res., 13:8913-8926 (1985)) and the like are known as subtilisins produced by bacteria of genus *Bacillus*. The signal peptides from them can be preferably used for the present invention although their amino acid sequences slightly vary each other. Various promoters which function in a bacterium of genus *Bacillus* can be used in place of the promoter from the subtilisin E gene which is used in the present invention for controlling expression.

The paragraph beginning at page 33, line, has been amended as follows:

The molecular weight of the mature Protease PFUS obtained from *Bacillus subtilis* DB104/pNAPS1 can be precisely measured, for example, by using a mass spectrometer. It is found from the measured molecular weight and the N-terminal amino acid sequence of the mature Protease PFUS determined as described above that the protease is a polypeptide corresponding to Ala at position 133 to Thr at position 552 of the amino acid sequence as shown in the SEQ ID NO:156 of the Sequence Listing.

Furthermore, a plasmid which expresses Protease PFUS lacking a polypeptide nonessential for its enzymatic activity can be constructed by introducing a termination codon in the vicinity of the portion encoding Thr at position 552 in the Protease PFUS gene contained in the plasmid pNAPS1. Specifically, a DNA fragment having a base sequence into which the intended termination codon is introduced can be obtained by PCR using the primer NPR544 which can introduce a termination codon (TGA) on the C-terminal side of the 544th amino acid residue encoding codon from the initiation codon in the Protease PFUS gene in the plasmid pNAPS1 (Ser) (the base sequence of the primer NPR544 is shown in the SEQ ID NO:28 of the Sequence Listing) and the primer NPFE81 which has the base sequence of the region upstream from the NspV site in the gene (the base sequence of the primer NPFE81 is shown in the SEQ ID NO:29 of the Sequence Listing). A mutant plasmid containing the protease gene into which the mutation of interest is introduced can be then obtained by replacing the fragment for the corresponding region in the plasmid pNAPS1. This plasmid is designated as the plasmid pNAPSΔC. *Bacillus subtilis* DB104 transformed with this plasmid is designated as *Bacillus subtilis* DB104/pNAPSΔC.

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methylcoumarin).

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The paragraph beginning at page 49, line 7, has been amended as follows:

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PFUS.

The paragraph at the bottom of page 53, has been amended as follows:

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The paragraph on page 53, line 13, has been amended as follows:

Mass spectrometric analysis on the purified enzyme preparations NAPS-1 and SPO-124ΔC was carried out using API300 quadrupole triple mass spectrometer (Perkin-Elmer Sciex). Based on the estimated molecular weight of NAPS-1, 43,744 Da, it was demonstrated that the mature Protease PFUS produced by *Bacillus subtilis* DB104/pNAPS1 is an enzyme consisting of a polypeptide from Ala at position 133 to Thr at position 552 of the amino acid sequence of Protease PFUS as shown in the SEQ ID NO:~~15~~16 of the Sequence Listing. Furthermore, based on the estimated molecular weight of SPO-124ΔC, 42,906 Da, it was demonstrated

that the mature Protease PFUS produced by *Bacillus subtilis* DB104/pSPO124ΔC is an enzyme consisting of a polypeptide from Ala at position 133 to Ser at position 544 of the amino acid sequence of Protease PFUS as shown in the SEQ ID NO:~~15~~16 of the Sequence Listing, i.e., the amino acid sequence as shown in the SEQ ID NO:~~2~~1 of the Sequence Listing.